CHAPTER 5

The preclinical development of (recombinant human) deoxyribonuclease I as a powder for inhalation

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ABSTRACT

A formulation and process development study was performed to formulate recombinant human deoxyribonuclease I as a powder for inhalation. First, excipient compatibility (with bovine DNase as a model substance) was examined with a stability study at stressed conditions (60 and 85 °C) while monitoring for occurrence of the Maillard reaction. Next, powders for inhalation were prepared by spray drying and spray freeze drying. We found that spray drying with inulin as stabilizer resulted in the best powder for inhalation. Finally, an ex-vivo test with the spray dried rhDNase I/inulin powder significantly decreased elastic and viscous moduli of sputum from 5 cystic fibrosis patients.

INTRODUCTION

Pulmozyme® is a solution for nebulization containing 2.5 mg/mL recombinant human deoxyribonuclease I (rhDNase I). RhDNase is used to decrease the viscoelastic properties of sputum in cystic fibrosis (CF) patients which results in an improved lung function (1, 2). In CF, repeated respiratory tract infections lead to high concentrations of DNA from degenerating polymorphonuclear leucocytes in the lung (3, 4). The presence of DNA increases the viscosity of sputum and makes it more difficult to clear the sputum from the respiratory tract. This increases the susceptibility to infection which, in turn, provokes an inflammatory response. The cycle of infection, inflammation and obstruction leads to progressive destruction of lung tissue and reduced life expectancy (5, 6).

Pulmozyme is one of the therapeutic options in CF. In a double-blind, randomized, placebo-controlled phase III study, which involved 51 CF centers and 968 CF patients, patients were randomized to receive 2.5mg rhDNase once or twice daily, for 6 months (7). Administration of rhDNase either once or twice daily reduced the risk of a respiratory infection requiring antibiotics by 22% and 34%, respectively. At the end of the 6-month period the improvement in forced expiratory volume in 1 second (FEV1) was 0% for the placebo group, 5.8% for the once-daily group, and 5.6% for the twice-daily group, showing that both the incidence of respiratory tract infections and lung function is improved by rhDNase.

Pulmonary administration of drugs by nebulization is a process with several drawbacks. One of the major drawbacks is low deposition efficiency, especially with traditional nebulizers which for instance have high residual volumes. For Pulmozyme, the pulmonary deposition was reported to range between 7.8% and 31.2% (8). Furthermore, the nebulization (including cleaning of the equipment) is time consuming and requires complex equipment using electricity or compressed air. Finally, the solution for nebulization has to be stored in the refrigerator (2-8°C). All these aspects reduce patient compliance to therapy and may reduce the success of rhDNase therapy. The development of a stable dry powder inhalation formulation containing rhDNase may improve the administration. Major advantages are the potentially higher lung deposition (which would also reduce costs), a reduced administration time and no need to store the rhDNase solution at low temperatures to prevent degradation (8-12). These advantages may results in an improved compliance which positively affects the therapeutic outcome.

Major aspects that have to be taken into account when a protein containing dry powder for inhalation is to be developed are:

- the excipients in the formulation should stabilize the protein during drying and subsequent storage,
- the powder characteristics should, in combination with the used inhalation device, allow for the generation of an aerosol with an aerodynamic particle diameter within the 1 to 5 µm range (preferably 2 µm) (13).
- ex vivo experiment should guarantee the activity of the protein in the therapeutic setting.

Drying of pure proteins often leads to partial or total inactivation due to degradation. However, recently several technologies have been described that allow for the production of a dry and stable powder formulation of proteins for inhalation (14-17). To withstand the degradation during processing and storage, stabilizing excipients should be used (18-21). The most common types of protectants are carbohydrates or polyols (e.g., mannitol, sucrose, trehalose, inulin). Spray (freeze) drying of aqueous solutions of rhDNase has been reported previously as a successful drying technique (14, 16). Maa *et al.*(16) have compared spray drying with spray freeze drying and found that with their inhaler (Dryhaler®) spray freeze drying was better suited to produce a rhDNase powder for inhalation. However, their findings may be rather specific for the used inhaler and spray freeze drying, in contrast to the spray drying, is difficult to upscale to an industrial process (22).

Quan *et al.* (23) have demonstrated that rhDNase is prone to the Maillard reaction when intimately co-formulated with reducing sugars, such as lactose. Hence, reducing sugars should be avoided as excipient for proteins in spray dried and freeze dried formulations. In a different study, Chan *et al.* (14) have spray dried pure rhDNase solutions and subsequently mixed the powder with lactose carrier, thereby keeping the number contact points between rhDNase and lactose low. However, for long term storage, a glassy formulation may be preferred.

The purpose of this study was to investigate whether spray drying of a (rh)DNase powder for inhalation with the Novolizer® dry powder inhaler is feasible. In this study the performance of spray dried DNase powder is evaluated in comparison with freeze dried and spray freeze dried powders regarding stabilization effectiveness, maintenance of enzymatic activity and dimerization of (rh)DNase. In contrast with previous studies, the

aerosol generation of the differently produced powders was studied without mixing the powders with additional excipients (e.g. crystalline lactose) to improve dispersion. Working without additional excipients reduces the amount of material to be inhaled. Finally, an ex vivo test, measuring the effect of rhDNase powder on the viscoelasticity of CF sputum, was performed.

MATERIALS AND METHODS

Materials

Pancreatic bovine deoxyribonuclease I (DNase, ~3000 units per mg, purity >80%) was purchased from Sigma-Aldrich. DNase was used as a model drug, since bovine DNase has a homology of ~80% in its amino acid sequence compared to rhDNase (24). DNase was the test drug for the entire study, with the exception of the ex vivo proof of principle, where recombinant human deoxyribonuclease I (rhDNase) was used, which was obtained as Pulmozyme® from Roche (Basel, Switzerland). Inulins with a degree of polymerization (DP) of 14 and 23 were a generous gift of Sensus (Roosendaal, the Netherlands). All other chemicals were either of reagent or of analytical grade and purchased from commercial suppliers.

Methods

Powder preparation methods

For all processes used to produce bovine DNase powders, the starting solutions were made in the same way. Each solution contained 2.5 mg/ml DNase, 0.375 mg/ml $CaCl_2$ and 11.0 mg/ml NaCl and the pH was adjusted to 6.3 with 0.2N HCl. $CaCl_2$ was added to the DNase-solution because it has been reported that this salt stabilizes two disulfide bridges in the protein (23). NaCl was added to ensure that DNase remained in its tertiary structure in the solution. Carbohydrates were added to the DNase solution in a final concentration of 22.5 mg/ml before drying.

Spray drying

Sucrose, trehalose, inulin DP14 and inulin DP 23 were initially tried in the spray drying process. For the inhaler experiments inulin DP23 was used as stabilizer. The solution was spray dried with a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a two-fluid nozzle (0.5 mm). The process conditions were: pump 2.37 mL/min (setting 3), aspirator 75 mbar (setting 18), heating inlet temperature 135 °C, outlet

temperature between 55 - 75 °C (setting 9) and the atomizing airflow was set at 400, 600 or 800 L_n/h. The obtained powder was subsequently stored at 20 °C and 50% relative humidity (RH).
To spray dry rhDNase, Pulmozyme® was used as starting material and the preparation of the solution was adapted to obtain similar concentrations as during DNase experiments. Firstly, Pulmozyme® was centrifuged with an Ultrafree© centrifugal filter device with a molecular weight cut-off of 10 kD (Millipore Corporation, Bedford, MA 01730) to obtain a more concentrated solution. After centrifugation, the concentration of rhDNase was measured with a Lowry assay (25). The $CaCl_2$ concentration was adjusted to the rhDNase concentration with microdialysis using a pH of 6.3. Afterwards, inulin DP23 was added to obtain a ratio of rhDNase:inulin of 1:9. The total concentration of inulin and rhDNase was 40 mg/mL and contained 0.6 mg/mL $CaCl_2$ at a pH of 6.3. The solution prepared in this way was similar to the solutions used for bovine DNase, only the concentration was slightly higher because centrifugation led to higher rhDNase concentrations. A similar solution was prepared without rhDNase to obtain a placebo powder.

Freeze drying
Sucrose, trehalose, inulin DP14 and DP 23 were used as stabilizers in the freeze drying process; a sample without carbohydrate was produced as reference material. The sample without carbohydrate thus only contained DNase (2.5 mg/ml), $CaCl_2$ (0.375 mg/ml) and NaCl (11.0 mg/ml). Freeze drying was performed using a Christ model Alpha 2-4 lyophilizer (Salm en Kipp, Breukelen, the Netherlands). For the freeze drying experiments, 4 mL glass vials were filled with 400 µl aqueous solution. The aqueous solutions were frozen in liquid nitrogen and subsequently dried at a condenser temperature of -53 ºC and a shelf temperature of -35 ºC at a pressure of 0.220 mbar. After 21 hours the shelf temperature was increased incrementally over 3 hours to 20 ºC and the pressure was decreased to 0.060 mbar. Secondary drying was continued for another 24 hours under these conditions.
After lyophilization the dried samples were transferred to a vacuum desiccator and stored for at least 1 day before using the samples in experiments.

Spray freeze drying

For the spray freeze drying process (with inulin DP23) the solution was sprayed into a bowl of liquid nitrogen with the two-fluid nozzle (diameter 0.5 mm). The same nozzle as used in the Büchi Mini Spray Dryer. The nozzle was placed approximately 10 cm above the surface of the liquid nitrogen and the solution was atomized with an atomizing air flows of 400, 600 and 800 L_n/h at a pump flow 2.37 mL/min. After the spraying was completed, the bowl with liquid nitrogen was transferred to a Christ model Alpha 2-4 freeze dryer (Salm & Kipp, Breukelen, The Netherlands) and the liquid nitrogen was allowed to evaporate. For drying, exactly the same regime was used as described for the freeze dried samples The obtained powders were subsequently stored at 20 °C and 50% RH.

Stability testing of the powders

Freeze dried samples were transferred to a vacuum desiccator for at least one day before using the samples in experiments. Long term storage of the freeze dried samples was at 0% RH and two different temperatures: 60 and 85 °C. The climates were controlled with a Hygrocontrol temperature and humidity transmitter (Hygrocontrol, Hanau, Germany).

DNase activity

The effect of freeze drying in the absence or presence of carbohydrates followed by storage at elevated temperatures on the enzymatical activity of DNase was determined as described by Sinicropi (26). Samples were reconstituted with 2.5 mL of HEPES buffer (containing 25 mM HEPES, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1% BSA, 0.01% thimerosal and 0.05% Tween 20) and diluted to DNase concentrations of 2000 ng/mL, 1000 ng/mL and 500 ng/mL. A calibration curve of unprocessed DNase was simultaneously prepared in HEPES buffer with a concentration range of 0-1000 ng/mL. The substrate was prepared by mixing 8.47 mL buffer A (25 mM Hepes and 1 mM EDTA, pH 7.5) with DNA from salmon testes (Sigma), 0.42 mL buffer B (20 mM acetate-NaOH, pH 4.2) containing 0.4% methylgreen and 2.21 mL buffer C (25 mM Hepes, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0,1% BSA, 0,01% thimerosal, 0,05% Tween 20, pH 7.5).

Next, the wells of a 96 wells plate were filled with 100 µl of the diluted sample and 100 µL substrate was added. The reaction was performed at room temperature. After 90 minutes the reaction was quenched by adding 50 µL of a solution containing 50 mM EDTA and 50 mM H_2O_2. After another

90 minutes the absorption was measured with a Biorad Microplate Reader Benchmark (Biorad, Hercules, United States) at 630 nm. The difference in absorption between 5 and 180 minutes was used to calculate the activity of the samples relative to unprocessed DNase.

Glass transition temperature

The glass transition temperature of freeze dried samples was determined with modulated differential scanning calorymetry (mDSC; DSC2920 differential scanning calorimeter, TA Instruments, Ghent, Belgium). The sample was preheated at 40 °C for 15 minutes and then heated to 200 °C at a rate of 2 °C/min and a modulation of ±0.318 °C/min. The midpoint of the deflection in the reversing heat flow – temperature curve was determined as the T_g. During the measurements, the cell was purged with nitrogen (35 mL/min).

Reducing groups

The amount of reducing groups in the carbohydrates was determined with the Sumner assay (27). In short, 100 mL of a solution with 20 g NaK-tartrate tetrahydrate, 1 g dinitrosalycylic acid, 1 g NaOH and 200 mg phenol was prepared. Subsequently, 1.2 mL of this solution was added to 0.8 mL of the carbohydrate solution. To this solution, 80 µL of a freshly prepared solution of 3 g Na_2SO_3 in 100 mL demineralised water was added. The resulting mixture was immediately vortexed and placed in a water bath of 95 °C. After 15 minutes the samples were removed from the water bath and allowed to cool down to room temperature. Finally, the absorbance of 200 µL was measured at 630 nm using a Biorad Microplate Reader Benchmark (Biorad, Hercules, United States). A calibration curve of glucose in a concentration range of 0.09 – 0.9 mg/mL was used in all experiments. The number of reducing groups was expressed as the percentage of sugar units containing reducing groups.

Monitoring of the Maillard reaction

The Maillard reaction occurs in three steps. In the first step the reaction between the carbonyl and free amino groups leads to a condensation product N-substituted glycosylamine, which transforms into the Amadori rearrangement product (ARP). Under acidic conditions, the ARP undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or 5-hydroxy-2-methylfurfural (when hexoses are involved). In

the intermediate step colorless Maillard intermediates are formed, such as 5-hydroxymethylfurfural and pyrazines. In the last step of the Maillard reaction the advanced glycation end-products are formed which can be noticed by their brown color (nonenzymatic browning) (28). The formation of 5-hydroxymethylfurfural and pyrazynes was investigated by measuring the absorbance of reconstituted samples at 280 nm using a Unicam UV 500 UV-VIS spectrophotometer (Thermospectronic, Cambridge, UK) (29, 30).

Dimeric aggregation

Size exclusion chromatography was performed on all DNase-carbohydrate samples stored for 0, 6, 14, 21 and 28 days at 85 °C. Prior to analysis, samples were dissolved with 1.0 mL HEPES buffer (as described under DNase activity) and filtered using a 0.45 µm filter. Subsequently, all solutions were placed in a Perkin-Elmer 200 series equipped with a Bio-Sil SEC250 column and a Bio-Sil 250 guard. As mobile phase 0.1 M sodium phosphate and 0.1 M sodium sulphate with a pH of 6.8 was used at a flow rate of 1.0 mL/min. For each run 20 µL sample solution was injected . Detection was performed with an ultraviolet detector (Applied Biosystems model 785A) at 215 nm. For calibration a GPC standard (Bio-Rad Laboratories, Hercules, USA) was used which contained bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa). The molecular weight of DNase is 31 kDa.

Scanning electron microscopy

Scanning electron micrographs (SEM) were recorded with a JEOL JSM 6301-F Microscope (JEOL, Japan). The powder was dispersed on top of double-sided sticky carbon tape on metal disks and coated with 150 nm of gold/palladium in a Balzers 120B sputtering device (Balzers UNION, Liechtenstein).

Laser diffraction analysis

Particle size distributions of the powders were measured in duplicate (the difference between the duplicates was less than $\pm$1% for all samples) with a HELOS Compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany: 100 mm lens and the Fraunhofer theory)

equipped with a RODOS disperser. The powders were dispersed at a pressure of 3 bar and the median volume diameters (X_{50}) were calculated.

Cascade impactor analysis

Cascade impactor analysis was carried out with a glass constructed four stage liquid impactor of the Fisons type (Elgebe, Leek, The Netherlands). The Novolizer® inhaler was attached to a dry bent induction port using a coupling flange with rubber seal ring. A solenoid valve was used in combination with a timer to control the flow rate through the inhaler and the cascade impactor for the duration of 3 seconds. The airflow through the inhaler, corresponding with a pressure difference of 4 kPa, was 71 L_n/min. The amount of drug deposited on the different stages was determined by the Lowry assay (25). Deposition was expressed as a percentage of the metered dose. The fine particle fraction (FPF), defined as a percentage of particles with an aerodynamic diameter less than 5 µm, was calculated by intrapolation of the cumulative mass plot versus effective cut-off diameter and expressed as a percentage of the metered dose. The mass median aerodynamic diameters (mmad's), were calculated from the cumulative mass percentages derived from the impactor stages 2, 3 and 4 (31), using the theoretical cutpoints for the different impactor stages.

Cascade impactor measurements were performed in triplicate.

Ex vivo rhDNase activity analysis

Approval for the collection of sputum of CF patients was obtained from the ethics committee of the University Hospital of Ghent. The sputum was expectorated spontaneously by CF patients (n=5) during chest physiotherapy sessions. After collection, sputum samples were immediately frozen at -20 °C (32, 33). The prepared placebo and rhDNase powder was mixed in a 1:10 ratio with sucrose to obtain a weighable dose of rhDNase. In total, rhDNase-dose was 30 µg (15 IU).

After thawing, a single sample of sputum was divided into 4-6 portions, depending on the volume of the sample. First, the viscoelasticity of untreated sputum was determined as 100% reference. Next, 30 mg of placebo powder or rhDNase powder was added and the viscoelasticity of the sputum was determined after 20 minutes of incubation at room temperature. This procedure was performed in such a way that at least

duplicate measurements were determined on a single sputum sample of placebo and DNase powder.
Sputum viscoelasticity was measured with a controlled-stress rheometer (AR 1000-N; TAInstruments, Ghent, Belgium) at 20 °C, using a cone-plate geometry. The angle between the cone and the plate was 2 degrees, and the sample volume required was approximately 0.9 mL. Dynamic oscillatory measurements were made at a constant frequency of 1 Hz, with a stress ranging from 0.01 to 0.1 Pa. To avoid disruption of the weak biopolymer network in sputum, the elastic (G') and viscous (G") moduli of the sputum samples were determined in the linear viscoelastic region.

RESULTS

Excipient compatibility and stability

To determine the effect of different excipients on the stability of bovine DNase the material was freeze dried in the presence of the four different carbohydrates. Figure 1 shows that freeze drying reduces the activity of bovine DNase by approximately 20% when no carbohydrate is present in the formulation. Drying in the presence of a carbohydrate reduces the activity loss to about 10%.
When stored at 60 °C all carbohydrates protected DNase from further loss of activity for a period of 6 weeks, whereas the DNase freeze dried without carbohydrate lost another 40% of activity within the same period. The type of carbohydrates did not seem to have a substantial effect at this storage temperature.

Table 1. Glass transition temperature and the percentage of reducing groups found for the different carbohydrates (freshly prepared freeze dried samples, n=3).

Sample	T_g (°C)	Reducing groups (%)
Sucrose	76.1 ± 1.1	0.09 ± 0.01
Trehalose *	121.6 ± 0.7	0.14 ± 0.04
Inulin DP14 *	139.7 ± 1.7	2.1 ± 0.1
Inulin DP23	157.1 ± 1.3	5.2 ± 0.2

* Data from Hinrichs *et al.* [21]

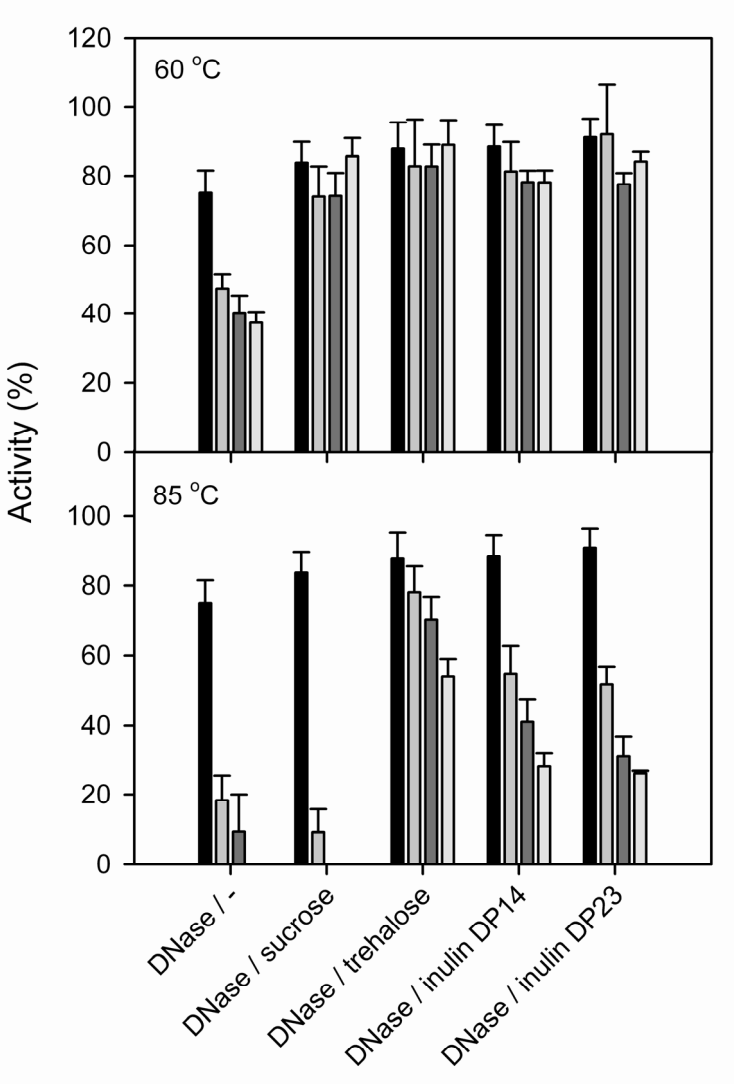

Figure 1. DNase activity of the different formulations after freeze-drying and storage at 60 °C or 85 °C. Storage times were 0 days (black bars), 2 weeks (grey bars), 4 weeks (dark grey bars) or 6 weeks (light grey bars). Results are expressed as averages (n=3) ± standard deviation.

When stored at 85 °C, DNase freeze dried without carbohydrates rapidly lost all of its activity. The sucrose formulation lost its activity even faster under these conditions. However the degradation rates of the trehalose and inulin formulations were clearly reduced compared to the reference

and the sucrose formulation. The formulations with inulin showed a faster loss of activity than the trehalose formulation, despite the higher Tg's of inulin (Table 1). In a control experiment, the presence of NaCl in the formulations did not change the T_g of the pure carbohydrate (data not shown). Visual observation of the stored cakes showed a dramatic volume contraction and browning, which was dependent on storage time. This effect was most pronounced for the sucrose-containing formulation stored at 85 °C. At this temperature, the sucrose formulation was stored above its Tg.

The Sumner assay revealed that sucrose and trehalose contain only minor levels of reducing groups whereas somewhat higher levels were found in the inulins (Table 1).

The intrinsic absorbance of DNase at 280 nm in all formulations immediately after freeze drying (t = 0) was found to be 0.18 (Figure 2A). During storage at 85 °C, the absorbance increased dramatically for the sucrose formulation, indicating a dramatic increase in the formation of 5-hydroxymethylfurfural and pyrazines (intermediates of the Maillard reaction). The increase was much less pronounced for both inulins, whereas the trehalose formulation showed no significant increase in absorption at 280 nm.

Upon reconstitution, approximately 10% of DNase was found as dimeric aggregates immediately after freeze drying, irrespective of the formulation (Figure 2B). During storage at 85 °C, the sucrose formulation resulted in the highest increase in the amount of dimeric aggregates. Also, the inulin formulations and the carbohydrate-free DNase showed a slight increase in the amount of dimeric aggregates during storage whereas this increase for the trehalose formulation was almost negligible.

Drying processes

In this study spray drying was chosen as process of first choice for the production of a dry DNase powder for inhalation. However, when spray drying was performed it was found that the sucrose formulation can not be processed properly at the conditions tested in our study. The particles immediately coalesced in the collection vessel. The trehalose and inulin formulations could successfully be spray dried but the trehalose formulation could not be stored adequately at ambient conditions (20-25 °C and 40-60% RH), due to coalescence of the particles, rendering an unsuitable formulation for inhalation. The inulin formulations showed

no coalescence during storage at ambient conditions, but in further spray drying experiments, we decided to investigate only the inulin DP23 formulation. The enzymatic activity of both the bovine DNase as well as of the rhDNase was found to be around 80% after spray drying.

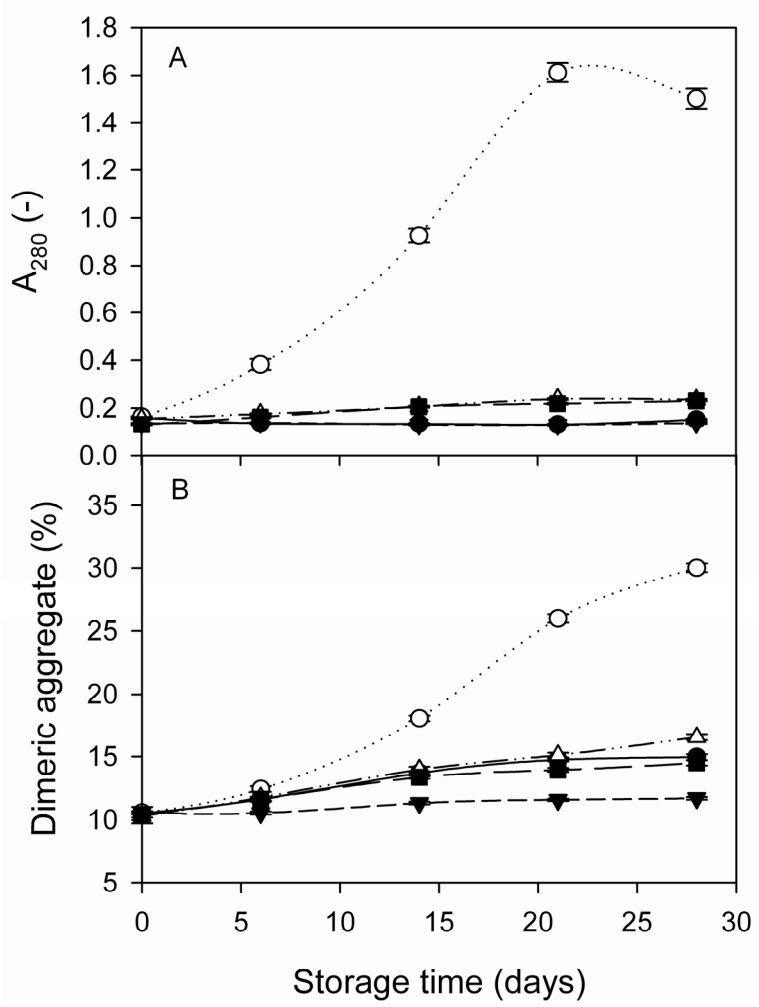

Figure 2. A: UV absorption at 280 nm of reconsituted freeze dried formulations after storage at 85 °C. B: percentage dimeric aggregates as found with size exclusion chromatography of reconstituted freeze dried formulations after storage at 85 °C: pure DNase (●), DNase-sucrose (○), DNase-trehalose (▼), DNase-inulin DP14 (■) and DNase-inulin DP 23 (△). Results are expressed as averages (n=3), error bars represent standard deviation.

Because inulin was the only carbohydrate to be processed successfully with spray drying, inulin (only DP23) was also selected for the preparation of a spray freeze dried powder in order to investigate the effect of process (spray drying vs. spray freeze drying) on the aerosol formation properties in inhalation experiments.

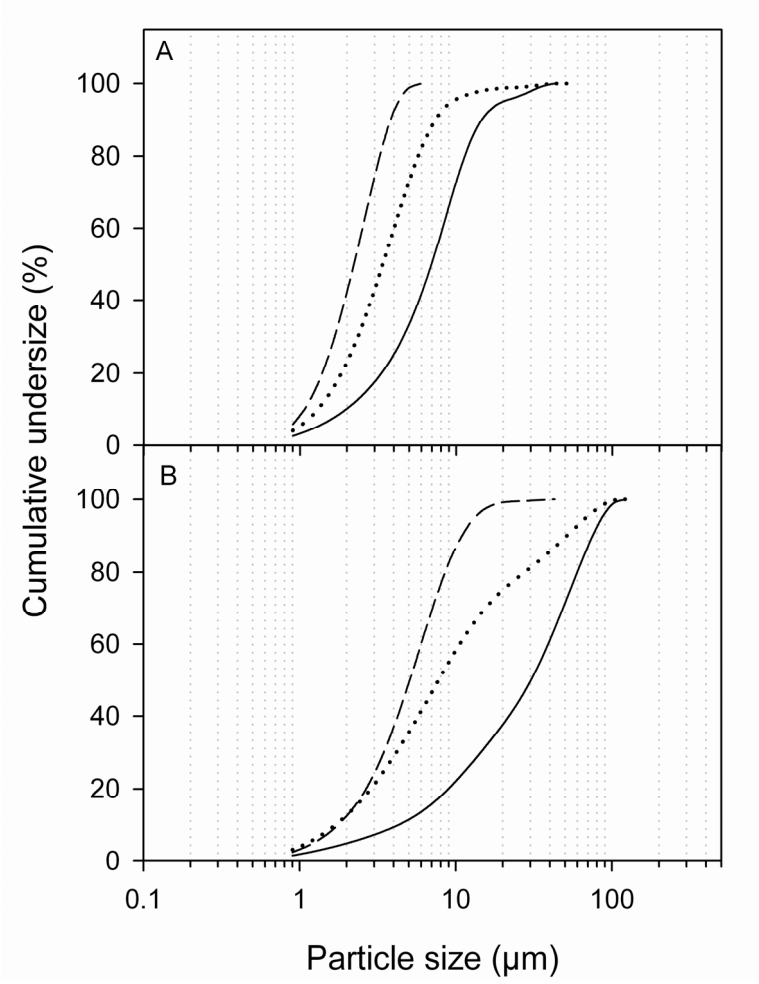

Figure 3. Effect of atomizing air flow on the powder particle size distribution of the DNase-inulin DP23 formulation after spray drying (A) or spray freeze drying (B) as determined by laser diffraction. Solid line represents 400 L_n/h, dotted line 600 L_n/h and crossed line 800 L_n/h.

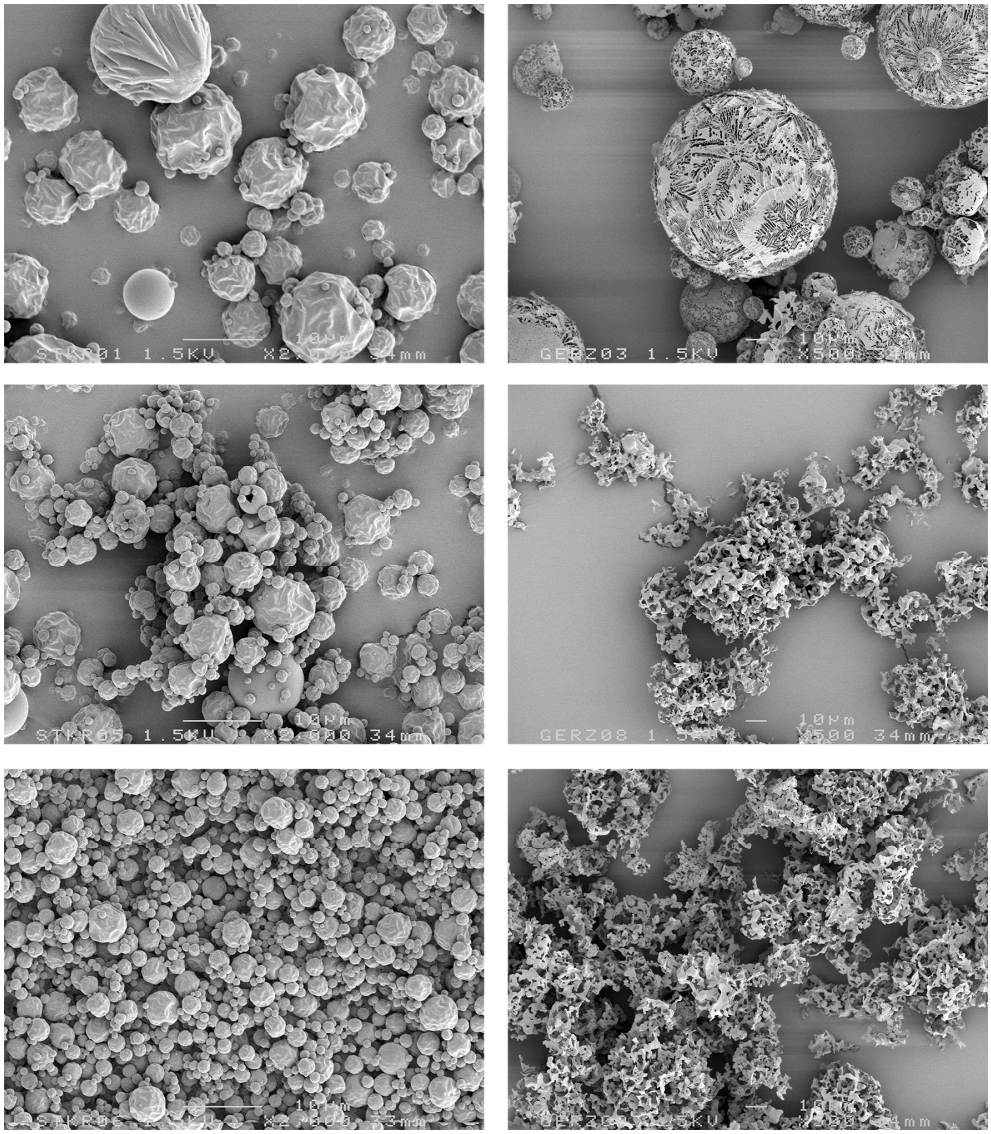

Figure 4. Scanning electron micrographs of spray dried (left) or spray freeze dried (right) of the DNase-inulin DP23 formulation. From top to bottom: atomizing air 400 L_n/h, 600 L_n/h and 800 L_n/h, respectively. Magnification factor of the spray dried formulation was 2000x and for the spray freeze dried formulation 500x.

Physical characteristics and in vitro deposition of the powders

Laser diffraction experiments revealed that spray drying at a higher atomizing air flow results in smaller particles (Figure 3A). The same effect was also found for spray-freeze drying (Figure 3B). Furthermore, spray freeze drying produced larger particles than spray drying at similar atomizing air flows (see also table 2). The morphologies of the particles obtained by spray drying and spray freeze drying are shown in Figure 4. Spray drying resulted in basically round, slightly dimpled particles. Spray freeze drying resulted in larger particles. At an atomizing air flow of 400 L_n/h, round intact spheres were produced, while at 600 and 800 L_n/h, the particles consisted of fragments of these spheres. These fragments tended to agglomerate and form larger "secondary" particles.

To determine the fine particle fraction from a marketed inhaler (Novolizer®) we performed a series of cascade impactor analyses with the spray dried powders. Figure 5A shows that a significant fraction of all three samples produced at different processing conditions deposited in the inhaler and induction port. Formulations prepared at increasing atomizing air flow showed an increased FPF.

From the cascade impactor data, mass median aerodynamic diameters were calculated (Table 2). Spray drying at higher atomizing air flows resulted in a powder having a smaller aerodynamic diameter (d_a). The mass median aerodynamic diameters of the powders prepared by spray drying and spray freeze drying at 600 L_n/h differed substantially, despite the fact that the same solutions and nozzles were used to produce the powders (Table 2). Yet, the fine particle fractions of the spray dried and spray freeze dried powders were comparable, 39.2 and 41.8%, respectively The difference in terms of dispersion and aerosol behavior between the powders was considerable (Figure 5B). The spray freeze dried powder displayed less deposition in the inhaler and induction port. In the cascade impactor, the spray freeze dried powder deposited mainly on the first and second stage, whereas for the spray dried powder the highest fractions were recovered from the third and fourth stage. The difference in the size distributions within the fine particle fractions is reflected by the mass median aerodynamic diameters, being 2.31 µm for the spray dried and 3.70 µm for the spray freeze dried product (Table 2).

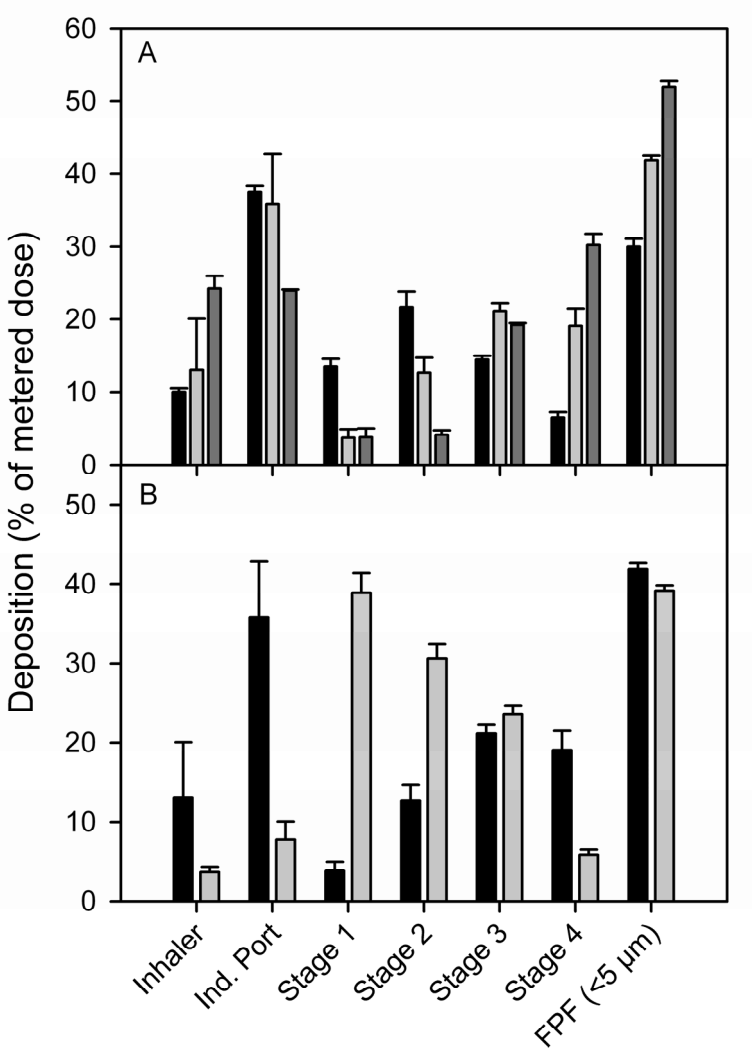

Figure 5. A: Cascade impactor analysis (4 kPa pressure drop) of the DNase-inulin DP23 formulation spray dried at 400 L_n/h (black bars), 600 L_n/h (grey bars) or 800 L_n/h (dark grey bars). B: Cascade impactor analysis (4 kPa pressure drop) of the inulin DP23 formulation spray dried (black bars) or spray freeze dried (grey bars) at 600 L_n/h. Results are expressed as averages (n=3) ± standard deviation.

Table 2. Particle size and aerosol performance of spray (freeze) dried DNase-inulinDP23 as a function of atomizing air flow and. All values are averages of duplicate measurements.

Production process	Atomizing air flow (L_n/h)	x_{50} (µm)	MMAD (µm)	FPF (%)
Spray drying	400	9.15 ± *0.38*	3.57 ± *0.01*	30.0 ± *1.1*
Spray drying	600	7.50 ± *0.92*	2.31 ± *0.10*	41.8 ± *0.7*
Spray drying	800	4.95 ± *0.55*	1.52 ± *0.02*	51.9 ± *0.8*
Spray freeze drying	600	18.67 ± *3.92*	3.70 ± *0.01*	39.2 ± *0.7*

X_{50}: median volume diameter
MMAD: Mass median aerodynamic diameter
FPF: fine particle fraction (< 5 µm)

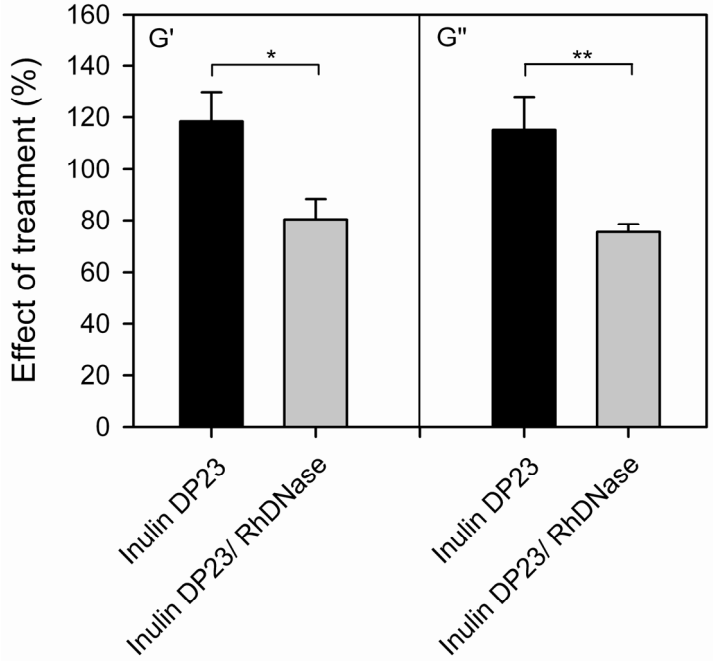

Figure 6. Effect of placebo (inulin DP23) or spray dried rhDNase-inulin DP23 formulation on elastic modulus (G') and viscous modulus (G") of CF sputum. Results are expressed as averages (n= 5) ± standard deviation. Statistics: Effect on elasticity of placebo versus rhDNase-inulin (*, p=0.025), effect on viscosity of placebo versus rhDNase-inulin (**, p=0.016).

Ex vivo rhDNase activity analysis

The effect of a spray dried rhDNase powder on the viscoelastic properties of sputum which was expectorated by 5 CF patients was determined (Figure 6). Addition of placebo powder to the sputum resulted in a 20% increase in elasticity (G') and viscosity (G") compared to the reference measurement. This increase is likely to be caused by partial evaporation of water from the sample due to the length of the measurement. However, addition of powder containing 15 Units rhDNase resulted in a significant decrease of around 40% compared to placebo of both the elasticity (G'; $p=0.025$) and viscosity (G"; $p=0.016$). This indicates that the rhDNase powder dissolved upon contact with the viscous sputum and that the reconstituted rhDNase was able decrease the viscoelasticity of the sputum. For Pulmozyme a similar effect has been described (6, 34).

DISCUSSION

In this study several major aspects related to the preclinical development of a rhDNase containing powder for inhalation were investigated.

Since the spray drying of the sucrose powders was not successful and trehalose rendered powders that showed rapid coalescence at ambient conditions, spray drying was not considered a suitable preparation method for the stability testing. Freeze drying is a suitable alternative since this process, like spray drying, produces powders in which contact between the protein and carbohydrate exists on a molecular scale. Moreover, it was found in this study that after both spray drying and freeze drying (for trehalose and inulin) DNase activity is still around 80%. To compare the effects of the four carbohydrates on the stability of DNase during drying and storage, freeze dried materials were stored under accelerated stress conditions. Fig. 2 shows that all four carbohydrates are suitable stabilizers for DNase at 60 °C. However, at the higher stress condition of 85 °C differences between the carbohydrates are found. The sucrose formulation led to extreme Maillard reaction when stored at 85 °C. This is explained by the fact that at 85 °C the sucrose system is in the rubbery state and the combination with the elevated temperature could have led to the formation of reducing degradation products which subsequently reacted with DNase. On the other hand, for the trehalose formulation no indication of the occurrence of a Maillard reaction was observed. Neither did the absorption at 280 nm increase nor did the amount of dimeric aggregates increase. The degradation found at

85 °C is only heat induced degradation of the DNase. The absence of any interaction of the DNase with trehalose is explained by the high glass transition temperature of the trehalose and the absence of reducing sugars in this material. Both inulin based products show some degradation at 85 °C. Although the degradation rates were considerable slower than in the sucrose product, they were somewhat faster than in the trehalose product. The small increase in the 280 nm absorption and amount of dimeric aggregates shows that the Maillard reaction is to some extent responsible for this degradation. Because of the high glass transition temperature of the inulins (139.7 °C and 157.1 °C for inulin DP14 and inulin DP23, respectively) it is unlikely that reducing degradation products are formed at 85 °C, which indicates that the reducing groups in the starting material are responsible for this degradation. In spite of the higher amount of reducing groups in the inulin DP23 there is no faster degradation compared to inulin DP14. This is explained by the higher glass transition temperature, since for amorphous systems, a low molecular mobility is favorable for storage stability (35).

The spray drying studies revealed another disadvantage of the low glass transition temperature of sucrose. It was found that the sucrose formulation can not be processed properly at the conditions tested in our study. The particles immediately coalesced in the collection vessel, most likely due to a relatively high temperature and residual moisture in the powder by which the Tg was passed. By optimizing the settings, conditions may be found with which the sucrose formulation can well be spray dried, but this was not investigated because of the poor performance of the sucrose in the stability study. The trehalose and inulin formulations could be successfully spray dried. However, the trehalose formulation could not be stored adequately at ambient conditions (20-25 °C and 40-60% RH), due to coalescence of the particles, rendering an unsuitable formulation for inhalation purposes. A similar finding was described by other authors (36). The inulin formulations showed no coalescence during spray drying and subsequent storage at ambient conditions. Based on this observation we decided to perform the further experiments with the inulin DP23 formulation. The inulin DP23 was chosen over the DP14 because of its higher glass transition temperature.

Overall it was concluded that sucrose is not a suitable excipient because of its low glass transition temperature and the formation of reducing degradation products that interacted with the DNase. The high glass

transition temperatures of trehalose and both inulins make these carbohydrates better suited as stabilizer for DNase. Although it would have been preferable that inulin starting materials are free from reducing sugars. On the other hand, the six weeks stability at 60 °C indicates that both trehalose and inulin powders can be stored for more than 1 year at room temperature in the complete absence of water without significant degradation.

During spray freeze drying droplets or fragments of the droplets are rapidly frozen in a rigid conglomerate of solid material and ice. Upon removal of the ice the original droplet dimensions are maintained. With spray drying, water evaporates from the surface of the droplet, resulting in droplet size reduction. At a certain point, a droplet surrounded by a rubbery shell is formed. Upon further drying, the pressure in the particle increases to such an extent that the shell disrupts and often collapses leading to rimpled particles. As a result, the size of the dry particle is often much smaller than the original droplet size. Since the solutions of which the droplets were made contained the same concentration of solid material irrespective of which drying process is used, it is obvious that the spray dried particles must have a higher apparent density. The SEM pictures and laser diffraction results show that an increasing atomizing air flow reduces the particle size when the material is spray dried. The smaller initial droplet size results in a smaller particle size (37).
The large fractions of fragments observed in the SEM pictures of spray freeze dried particles that were produced at atomizing air flows of 600 or 800 L_n/h indicate that the droplets are scattered upon impaction with the liquid nitrogen during the spraying process. Only at the lower atomizing air flow the droplets remain intact when sprayed into the nitrogen.

The cascade impactor experiments clearly show that the reduced particle size of spray dried powders produced with higher atomizing air flows is reflected in an increased fine particle fraction. Also the MMAD decreased for the particles produced at higher atomizing airflows. The particles produced at 400 L_n/h are certainly too large, since Geller *et al*. (13) have reported that nebulization of Pulmozyme in a droplet size distribution with a MMAD of 2.1 µm, resulted in larger improvement of FEV_1 than a droplet size distribution of 4.9 µm: 4.3% vs. 2.5%, respectively. On the other hand, the powder with the MMAD of 1.52 µm may be too small to obtain the desired effects (38). Therefore, the powder produced at 600

L_n/h which gives an MMAD of 2.31 µm from the Novolizer at 4 kPa seems preferable.
The larger MMAD found for the spray freeze dried powder compared to the spray dried powder is remarkable. Since the spray freeze dried powder is formed from the same droplets (or fragments thereof) as the spray dried powder, the same MMAD or a slightly lower MMAD is expected. The higher MMAD indicates that powder de-agglomeration is incomplete for the spray freeze dried powder. This observation is in contrast with the findings of Maa *et al.* (16) who concluded that aerosol performance was superior with spray freeze dried particles. The reason for the better performance of the spray dried particles in this study may be linked to the powder de-agglomeration principle in the Novolizer, which makes use of inertial forces (39, 40). Differences in particle density may cause a difference in classifier payload (at the same dose weight) and this, in combination with a difference in the rate and mode of agglomerate break-up, may result in different effective cutpoints for the classifier. These findings clearly show that drug development for inhalation does not stop at powder formulation, but that inhaler design plays an important role too. Most currently marketed inhalers are not suited for effective de-agglomeration of different types of inhalation powder. Besides, most inhalers do not perform well on powders in the micron range without processing them into adhesive mixtures.

Finally, exposure of the preferred powder formulation to sputum from CF patients resulted in decreased viscosity and elasticity. Sputum from CF patients is extremely viscoelastic because of a low water content and an enhanced presence of extra cellular DNA and actin from degenerating neutrophils, serum proteins, and lipids (4, 41-44). It can be assumed that the mechanism by which the viscoelasticity is decreased by the rhDNase powder is similar to that of the liquid formulation: enzymatic break down of the DNA polymer network and thereby reduction of the typical viscoelasticity of the mucus (2).

In summary, this study shows that a stable rhDNase powder for inhalation can be designed. The stabilizing carbohydrate should be non-reducing and have a high glass transition temperature. Furthermore, utilization of the Novolizer inhaler (which generates high inertial de-agglomeration forces) allowed for the use of the spray drying process to produce the particles

with a high fine particle fraction having a desired MMAD of 2.3 μm. Future research should be directed to patient studies to investigate whether the dry powder inhalation system of rhDNase would indeed increase lung deposition and better fulfill patients' needs in terms of improved ease of administration and compliance and whether this could reduce costs of the therapy (45).

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